PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 94/02635
C12Q 1/68	A1	(43) International Publication Date: 3 February 1994 (03.02.94)

(21) International Application Number: PCT/GB93/01559

(22) International Filing Date: 23 July 1993 (23.07.93)

(30) Priority data:
9215656.1 23 July 1992 (23.07.92) GB

(71) Applicant *(for all designated States except US)*: THE MINISTER OF AGRICULTURE FISHERIES AND FOOD IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Whitehall Place, London SW1A 2HH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WOODWARD, Martin, John [GB/GB]; 23 Burnsall Close, Farnborough, Hampshire GU14 8NN (GB). WEBSTER, Katherine, Anne [GB/GB]; 16 Mount Street, Dorking, Surrey RH4 3HT (GB).

(74) Agent: LOCKWOOD, P., B.; MOD (PE), DIPR, IPR1b Room 2002, Empress State Building, Lillie Road, London SW6 1TR (GB).

(81) Designated States: AU, CA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETECTION OF CRYPTOSPORIDIUM

(57) Abstract

An improved method is provided for diagnosis of cryptosporidiosis, an important zoonosis involving farm animals, using novel oligonucleotide probes and polymerase chain reaction primers specific to *C.parvum* and *C.baileyi*. Probes and primers which differentiate between the two are also provided. Ligase chain reaction (LCR) may also be applied. The specific oligonucleotide probes and primers of the invention can be applied to oocysts in samples such as surface water, faeces etc. when a preferred embodiment which breaks these open is applied.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NE**	Niger
BE	Belgium	**GN**	Guinea	**NL**	Netherlands
BF	Burkina Faso	**GR**	Greece	**NO**	Norway
BG	Bulgaria	**HU**	Hungary	**NZ**	New Zealand
BJ	Benin	**IE**	Ireland	**PL**	Poland
BR	Brazil	**IT**	Italy	**PT**	Portugal
BY	Belarus	**JP**	Japan	**RO**	Romania
CA	Canada	**KP**	Democratic People's Republic of Korea	**RU**	Russian Federation
CF	Central African Republic			**SD**	Sudan
CG	Congo	**KR**	Republic of Korea	**SE**	Sweden
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovak Republic
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LU**	Luxembourg	**TD**	Chad
CS	Czechoslovakia	**LV**	Latvia	**TG**	Togo
CZ	Czech Republic	**MC**	Monaco	**UA**	Ukraine
DE	Germany	**MG**	Madagascar	**US**	United States of America
DK	Denmark	**ML**	Mali	**UZ**	Uzbekistan
ES	Spain	**MN**	Mongolia	**VN**	Viet Nam
FI	Finland				

DETECTION OF CRYPTOSPORIDIUM.

The present invention relates to a method for the detection and/or identification of organisms and/or nucleic acid sequences of Cryptosporidium parvum (C.parvum) or baileyi (C.baileyi) and to oligonucleotides and test kits containing them for use in said method.

Cryptosporidosis is a parasitic disease caused by protozoans of the coccidial genus Cryptosporidium. In the UK, the first human infections were reported to the Communicable Disease Surveillance Service in June 1983 and between then and 1989 reported cases had risen to 8,019 per annum.

The disease has an incubation period of 1-14 days with symptoms lasting for 7-60 days and pathogenicity demonstrated mainly in a syndrome of diarrhoea of newborns and in cases of severe and prolonged gastroenteritis in immunodeficient patients. However, in people with a normal immune response the vast majority of cases involve acute self-limiting diarrhoeal symptoms. There is no chemotherapy, only supportive treatment can be offered.

The following species and respective hosts have been determined by cross-transmission studies:

C.parvum	mammals
C.muris	rodents (mice)
C.baileyi	avians
C.meleagridis	avians
C.crotali*	reptiles
C.nasorum*	fish

* = Not thought to occur in the UK.

Large numbers of oocysts have been recovered from waste, surface and

recreational waters, but their origin is unknown. These oocysts have been known to survive for considerable periods in water and are unaffected by conventional water treatments (Smith et al (1989) Parasitology 99:323-7).

It is known that cryptosporidiosis is a zoonosis and several human outbreaks have implicated bovine slurry runoff into water sources as a focus of infection, but the importance of this route is not fully understood as currently there is no adequate test to distinguish between different oocysts (Chermetie et al (1988) Cryptosporidiosis: 2nd edition. Technical series number 5. Office International des epizooties. Paris).

The present invention provides an improved method for diagnosis of cryptosporidiosis, an important zoonisis involving farm animals, and further provides oligonucleotide probes and polymerase chain reaction primers specific to C.parvum and C.baileyi and still further provides probes and primers which differentiate between the two.

The invention provides a method of detecting and identifying C.parvum and baileyi specifically by identifying DNA of SEQ ID No 7 or 8, or subsequences having SEQ ID No 1, 2, 5 or 6 described herein, by use of hybridization probing and/or nucleotide sequence amplification, eg by polymerase chain reaction (PCR) or ligase chain reaction (LCR). Amplification of these sequences by the PCR or LCR provides direct indication of positive result through observation of product formation of predicted size or confirmatory hybridization probing. The specific oligonucleotide probes and primers of the invention can be applied to suitably treated samples (eg. surface water, faeces etc.) not only to detect but also to differentiate these Cryptosporidium.

Probing will conveniently be by carried out by using radiolabeled probes in Southern Blotting, but use of other probing techniques is also provided, eg. by tagging oligonucleotides complementary to the

target sequence with a marker, eg. such as biotin or a fluorescent agent, and relating presence of bound marker with a positive result, eg. by using biotin tag as active agent in an ELISA in the known manner or detecting fluorescence.

The present invention particularly provides a method capable of the detection of C.parvum and C.bailevi oocysts in contaminated water, a task requiring extremely sensitive techniques. The specificity of the method of the present invention has been confirmed by testing for cross-reactivity with Giardia and Eimeria and other water-borne organisms and microbes.

Typically the specific sequence amplification primers of the invention are applied to crude preparations of oocysts, faecal or water samples, together with chain reaction reagents and the performance of the reaction is assessed under these conditions. Oocysts may be isolated by concentration from water according to the method of Musial and colleagues, and DNA released. Amplification using PCR or LCR can then be used to detect the presence of Cryptosporidium and differential hybridization probing or further PCR or LCR used to speciate them.

A first aspect of the present invention provides a method for the detection and/or identification of C. parvum organisms, or polynucleic acid specific thereto, comprising determining the presence of the nucleic acid sequence SEQ ID No 1, or its complementary sequence SEQ ID No 2, in polynucleic acid present in or derived from a sample under investigation, and relating the presence thereof to presence of C.parvum or polynucleic acid thereof.

In a preferred emodiment of this aspect of the present invention the method comprises hybridization probing polynucleic acid present in or derived from a sample under investigation with an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID No 1, or its complementary sequence SEQ ID No 2,

and relating the occurence of hybridization with the presence of C. parvum or polynucleic acid thereof. Such hybridization may be carried out by any of the conventionally known methods, eg. by Southern Blotting or one of the tagging methods referred to above.

The term 'characterisitic of' as used herein refers to sequences that have sufficient consecutive bases homologous with the sequence of interest to be considered statistically highly likely to be that sequence. Thus herein a 'characteristic sequence' is one identical to any 10 consecutive bases of the sequence of interest, in this case SEQ ID No 1 or 2. Preferred probes are of SEQ ID No 1 and/or 2.

The specificity of the probing is controlled in the known manner by selecting stringency conditions such that the chosen probe will not hybridize with sequences found in other organisms that are known to be present in or considered likely to be found in such sample. This can be determined by carrying out control experiments. Furthermore, probes can be confirmed as specific by comparing their complementary sequences to sequences found in other known organisms. Such comparison can be readily made using computer databases, such as EMBL or GENBANK, whereby rapid comparison of sequences can be made.

The present invention further provides polynucleotide probes suitable for this method, preferably comprising a polynucleotide of SEQ ID No1 and/or 2, said probe being optionally labelled in known manner, eg. by incorporation of a radioactive (eg. ^{32}P), chemical or biological label into its structure; most preferably consisting of these sequences.

In a preferred embodiment the present invention provides a method for the detection and/or identification of C.parvum organisms or nucleic acid specific thereto comprising use of specific sequence amplification, eg. the polymerase chain reaction or ligase chain reaction, to amplify SEQ ID No 1 and/or SEQ ID No 2 . The use of primers in the known manner may be made to target these sequences to

provide an all or nothing amplification indicative of the presence of C. parvum.

Thus, for example, for the polymerase chain reaction, forward and reverse PCR primers having 5' end sequences SEQ ID No 3 and SEQ ID No 4 may be used, with or without 5' end non-hybridizing extensions:

SEQ ID No 3	5' ACG TAG CGC CGG ACG 3'
SEQ ID No 4	5' ACG CGC TGC TGC TGT 3'

eg. consisting of these sequences or any other primers which specifically provide for PCR amplification of the C. parvum specific sequences SEQ ID No 1 and/or 2. It will be understood by those skilled in the art that for ligase chain reaction longer primers will be required, having a smaller gap between them on the target sequence.

In a second aspect the present invention provides a method for the detection or identification of C.parvum or C.baileyi organisms or polynucleic acid specific thereto, comprising determining the presence of the nucleic acid SEQ ID No 5 and/or 6, in polynucleic acid present in or derived from a sample under investigation, and relating the presence thereof to presence of one of these organisms or their polynucleic acid.

In a preferred embodiment of this aspect the method comprises hybridization probing polynucleic acid, present in or derived from a sample under investigation, with an oligonucleotide that is capable of specific hybridization with a sequence characteristic of sequences SEQ ID No 5 or the sequence complementary thereto, SEQ ID No 6, these being sequences of the Cryptosporidium genome in which SEQ ID No 1 and 2 are located in C. parvum; ie. bases 123 to 152 of SEQ ID 7 and the corresponding part of SEQ ID No 8 in the sequence listing attached. In the C. baileyi genome the SEQ ID No 1 and 2 are somewhat different and thus identifying sequences that consist of mainly of the

characteristic parts of these is not indicative of that organism. Thus for detection of both of these species, or positive identification of C.baileyi alone, the aforesaid sequences SEQ ID 1 and/or 2 should not be used unless part of larger probes used under less stringent hybridization conditions.

In a further aspect of the invention the method determines the presence of a SEQ ID No 7 and/or 8, as referred to above, and relates that to the presence of C.parvum or C. baileyi and or polynucleic acid of either. Preferably the method comprises hybridization probing with a sequence capable of specifically hybridizing with sequnces characteristic of these, as defined above, again not using those having SEQ ID No 1 and/or 2 to positively identify C.baileyi.

Specific hybridization may be carried out in each case with hybridization conditions stringent enough to exclude interfering organisms and genomic DNA from other Cryptospridium organisms while being of low enough stringency to allow hybridization with both the parvum and baileyi sequences. The determination of such conditions will be readily made by those skilled in the art by use of simple control experiments and the protocols outlined in the examples provided herein.

The present invention further provides a method for detecting or identifying C.parvum or C.baileyi organisms or nucleic acid sequences specific thereto comprising

(a) mixing a sample suspected of comprising said organisms and/or nucleic acid sequences with polymerase chain reaction or ligase chain reaction reagents, and respective primers targeted to specifically amplify a polynucleotide sequence within SEQ ID No 7 and/or 8 comprising polynucleotides of SEQ ID No 5 and/or 6;

(b) subjecting the mixture to conditions under which amplification of

any sequence comprising SEQ ID No 5 and/or 6 present will occur;

(c) relating the production of a polymerase chain reaction product corresponding in size to SEQ ID No 5 and/or 6 to the presence of C. parvum and/or C.bailyei organisms or nucleic acid sequences specific thereto.

Preferably the polynucleotide sequence amplified is the double stranded sequence found within SEQ ID No 7 and 8, shown between base 91 and 420 of SEQ ID No 7, or the equivalent sequence of C. baileyi wherein the sequences equivalent to SEQ ID No 1 and 2 are present. Thus the preferred polynucleic acid sequences amplified are those amplified by PCR primers of SEQ ID No 9 and 10 described in the sequence listing given herein.

Again, in order to ensure specific amplification of the target sequence it is convenient to determine the degree of specificity required of the primers to be selected by either using control experiments using DNA of organisms likely to be found in the samples to be tested as controls with which the selected primers should not initiate a polymerase chain reaction. In a further method of selecting primers the primer nucleotide sequence contemplated for use may be compared to known sequences on databases such a EMBL and Genbank where a finding of low homology with recorded sequences, particularly in the area of the primer 5' end sequence, indicates suitability for specific amplification.

Where verification of production of a PCR or LCR product or a native unamplified sequence as being one of C. parvum or C. baileyi is intended then probing may be carried out using a labelled probe specific for the sequences above eg. probes comprising any of the sequences SEQ ID No 1 to 8 or double stranded sequences containing these. Smaller probe sequences selected from the aforesaid sequences

will also be derivable by the person skilled in the art by use of control experiments and/or databases referred to above, but it is generally recognised that sequences of complementary to 10 or more consecutive bases of the target polynucleic acid will be specific enough in binding for verification of presence of such sequences.

Preferably, where PCR is used, the primers should be of 6 or more bases long, more preferably 10 or more, eg. 25 bases and most preferably from 10 to 20 bases long.

Particularly preferred PCR primers found to be specific for amplification of DNA characteristic of SEQ ID No 7 and/or 8 for the purposes of identifying a sequence including SEQ ID No 5 and/or 6 against a background of other organisms have 5' end SEQ ID No 9 (forward primer) and SEQ ID No 10 (rearward primer).

SEQ ID No 9 5' ATC TTC ACG CAG TGC GTG GT 3'
SEQ ID No 10 5' CAT CAG CCG GTA GAT GTC GA 3'

Most preferably the primer sequences consist of these sequences. The present invention further provides such pimers per se.

The unamplified or amplified polynucleotide sequence may be identified by hybridization probing using a suitably labelled oligonucleotide that is capable of specific hybridization with the sequence SEQ ID No 5 and/or 6 or larger sequences containing them, eg. SEQ ID No 7 and 8 or double stranded sequences made up of these.

Where it is required to determine the presence or identity of one of C.parvum or baileyi specifically they can be identified by use of a probe of for SEQ ID No 1 and/or 2 as described above; hybridization being indicative of C. parvum. The present invention further provides a method of determining the presence of C.parvum as opposed to C. baileyi by use PCR or LCR to amplify the sequences specific to

both and then applying PCR, LCR or hybridization probing to the product to determine the presence or otherwise of SEQ ID No 1 and/or 2, and relating that to presence of one or other of C. parvum or baileyi.

It will be understood by those skilled in the art that when any of the methods outlined above are being applied to whole organisms, rather than to nucleic acids derived from them, it will be necessary to incorporate a procedure for liberating nucleic acids from said organism before initiating amplification chain reaction conditions.

The preferred forms of the present invention provide particular procedures whereby the sample to be tested is first suspended in a medium, eg. a buffer, and then either sonicated or subjected to freeze/thaw cycles in buffer containing a reducing agent, eg. dithiothreitol (DTT), in order to rupture the organisms, particularly their oocyst forms, and liberate the polynucleic acids. Particularly preferred are formats that isolate oocysts using antibodies to oocysts that have been tagged with metallic particles such that use of magnetic fields can recover individual oocyst/antibody complexes.

Using these preferred methods of the present invention it is possible to achieve detection or identification of Cryptosporidium organisms in oocyst form or otherwise. Preferably the sonication or freeze/thaw procedure is carried out in a medium which will form all or part of the PCR, LCR or probing medium, eg. the 1xTE medium referred to below, together with a reducing agent, eg 1-2% DTT in buffer. Sonication is conveniently carried out using about 11 μm peak to peak. Alternatively, for freeze/thaw procedure, the sample containers can be conveniently immersed in a cryogenic liquid such as liquid nitrogen. Other methods for disrupting cell or oocyte structures to liberate nucleic acids into the PCR or LCR probing medium will be known to the skilled person and may be expected to be applicable before initiating the PCR, LCR and/or probing procedures.

In a still further aspect of the present invention there are provided test kits comprising one or more probes and/or primers of the present invention as described herein above, in addition to these probes and primers per se.

The method, primers and probes of the present invention will now be described by way of illustration only by reference to the following protocols and examples. Further variants of the method, primers and probes falling within the scope of the invention will occur to the person skilled in the art in the light of these.

EXAMPLE 1: POLYMERASE CHAIN REACTION DETECTION OF C. PARVUM NUCLEOTIDE SEQUENCES IN FORM OF ISOLATED DNA.

Primers SEQ ID No 9 and 10 were used in the following reaction mix/conditions to amplify an approximatetly 330 bp fragment from C.parvum and C.baileyi. These are 53F/53R in the priority document.

Reaction mix (volume 50 µl)

dntp,s	-250 µmoles of each
primers	-100 pmoles of each primer (SEQ ID 9 and 10)
glycerol	-5% v/v
Taq polymerase	-2.5 units

1XTE (10mm Tris.cl pH 8; 1mm EDTA pH8)

Buffer 5.5 mm $MgCl_2$
50mm KCl
10 mm Tris.cl pH 8.3

plus the target nucleic acid to be amplified in a volume of 1-10 µl.

Reaction Conditions

Initial denaturing step	94°C for 3 minutes
Denature	94°C for 90 seconds)
Anneal	50°C for 120 seconds) 40 cycles
Extend	72°C for 120 seconds)
Final extension	72°C for 5 minutes

The sample is then run on a 2% horizontal agarose gel, stained with ethidium bromide and viewed under UV illumination whereby the method has been shown to detect 30 fg (30×10^{-15}g) of relatively pure C. parvum DNA by comparison of the sample gel with those from positive and negative DNA controls. C.baileyi also gave an approximately 330 bp product using these conditions.

EXAMPLE 2. PCR HYBRIDISATION PROBING.

The DNA on the stained gel produced in Example 1 was transferred onto a nylon filter (trademark-Hybond N) by Southern blotting, denatured and hybridised with a ^{32}P radiolabelled (multiprime labelling procedure) cloned C. parvum internal probe SEQ ID No 1. After washing off non-specifically bound probe, the filter is exposed to X-ray film (autoradiography) for 12 hours or 7 days. Detection limits are respectively: 12 hours 0.3 fg, 7 days 0.03 fg of C. parvum DNA. It will be realised that the hybridization probe might be used directly on the sample DNA without PCR but that this will necessarily reduce sensitivity. C. baileyi derived DNA did not give a positive result in the hybridization probing with SEQ ID No 1.

Negative controls. DNA from a number of other organisms was been tested and proven negative both by visual examination of the PCR product on a gel and after hybridisation. Cryptosporidium muris; Giardia lamblia; Tritrichomonas foetus; Candida albicans; Eimeria spp mixed ovine culture; Staphylococcus aureus; Esherichia coli; Streptococcus bovis; Baccilus sp.; Actinobacter sp.; Pseudomonas sp.; Enterobacter sp.; Haemonchus contortus; Trichostrongylus sp.; Ostertagia sp.; DNA of bovine, murine and avian origin.

EXAMPLE 3. PREPARATION OF WHOLE OOCYSTS FOR PCR.

A rapid protocol was used for preparation of oocysts (the infective stage of Cryptosporidium) for PCR to avoid the long procedure

associated with preparation of DNA.

1. The sample to be tested is resuspended in a solution of 1X TE (see above); 1% dithiothreotol (approx final volume is 100µl) 2. The oocysts are broken open by a) 3 cycles of sonication for 10 seconds (11µm peak to peak) with a 50 second rest, or b) 3 freeze/thaw cycles in liquid nitrogen. 3) The sample is then heated to 90°C for 20 minutes. 4) Debris is pelleted by centrifugation at 13000g for 5 minutes. 5) 1-10 µl of the supernatant is used as the template for PCR.

A series of oocyst concentrations were used to determine the detection limits of the reaction. For C.parvum oocysts detection limits are: 2000 oocysts by visual examination of gel of PCR product; 20 oocysts following probing and autoradiography for 7 days.

Cross-reactivity with other isolates.

4 bovine isolates of C.parvum obtained from different areas of the UK were examined and the region encoded by the SEQ ID No 5 and 6 is conserved in each of these isolates.

EXAMPLE 4: APPLICATION OF METHOD TO HUMAN AND ANIMAL FAECAL SAMPLES.

Human faecal samples were obtained from 5 patients from three towns in Scotland; animal faecal samples were obtained from 15 sites in England.

Human faeces: each sample was diluted 1/10 and 1/100 in 1xTE/2%DTT buffer, freeze thawed in liquid nitrogen 90°C five times, heated to 90°C for 20 minutes, debris pelleted by centrifugation at 13,000g for 5 minutes and the supernatant used as template for PCR. The PCR method of the present invention was compared to A/P staining.

Results:

Sample	A/P STAIN	PCR(1/10)	PCR(1/100)
1	2+	+	+
2	-	+	+
3	1+	-	+
4	2+	+	+
5	2+	-	+

The results were obtained when the tests were carried out on three separate occasions, and these and other results suggest that 1/100 is the optimal dilution. Tests were carried out on animal faeces as above using 1/100 dilution but using the SEQ ID No 7 as a probe to determine PCR product identity:

Results:

Host	A/P STAIN	PCR(1/100)+SEQ ID No 7
Bovine	-	-
''	2+	+
''	2+	+
''	3+	+
''	+	+
''	-	-
''	+/-	-
Cervine	4+	+
Bovine	3+	+
''	3+	+
''	+	+
Ovine	-	+
Bovine	-	+
''	-	-
''	3+	+

- = negative
+ = 1 or 2 oocysts per slide
2+= 1 oocyst per field
3+= 2-5 oocysts per field
4+= 5-10 oocysts per field

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: THE MINISTER OF AGRICULTURE FISHERIES AND FOOD IN HER BRITA
(B) STREET: WHITEHALL PLACE
(C) CITY: LONDON
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): SW1A 2HH

(A) NAME: MARTIN JOHN WOODWARD
(B) STREET: 23 BURNSALL CLOSE
(C) CITY: FARNBOROUGH
(D) STATE: HAMPSHIRE
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): GU14 8NN

(A) NAME: KATHERINE ANNE WEBSTER
(B) STREET: 16 MOUNT STREET
(C) CITY: DORKING
(D) STATE: SURREY
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): RH4 3HT

(ii) TITLE OF INVENTION: DETECTION OF CRYPTOSPORIDIUM (iii) NUMBER OF SEQUENCES: 10
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS (D) SOFTWARE: PatentIn Release £1.0, Version £1.25 (EPO)
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9215656.1 (B) FILING DATE: 23-JUL-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACGTAGCGCC GGACGACAGC AGCAGCGCGT

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACGCGCTGCT GCTGTCGTCC GGCGCTACGT 30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACGTAGCGCC GGACG 15

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACGCGCTGCT GCTGT 15

[2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 240 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCGAAACCGT CAACGTAGCG CCGGACGACA GCAGCAGCGC GTGTTGATGG TCGGCACCGG 60

CCACGGCTGC ATCGACTTGA AGGCGAAACC AGCGCTGCCG GACCGTTGTT CGGCCACACA 120

GCCGAAAAAT CCGGCCAGAT CATTATTACT ACAATTGAAA TATCATTGCA AGAAACAGAT 180

CGGAGAAGAT TTCATAGATA ATTTTGAACA GCTAAGTAAT AATATATATT TAAAGACAAT 240

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 240 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATTGTCTTTA AATATATATT ATTACTTAGC TGTTCAAAAT TATCTATGAA ATCTTCTCCG 60

ATCTGTTTCT TGCAATGATA TTTCAATTGT AGTAATAATG ATCTGGCCGG ATTTTTCGGC 120

TGTGTGGCCG AACAACGGTC CGGCAGCGCT GGTTTCGCCT TCAAGTCGAT GCAGCCGTGG 180

CCGGTGCCGA CCATCAACAC GCGCTGCTGC TGTCGTCCGG CGCTACGTTG ACGGTTTCGC 240

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GATCTCTAGC CAACGTTGAC CACCTTCTTT TAATAGCCAA GTTAAAATAG CTCTGAATAT 60

TCTTGGAGCA GATTGAGCAA TAAAGTGAAG ATCTTCACGC AGTGCGTGGT GCGAAACCGT 120

CAACGTAGCG CCGGACGACA GCAGCAGCGC GTGTTGATGG TCGGCACCGG CCACGGCTGC 180

ATCGACTTGA AGGCGAAACC AGCGCTGCCG GACCGTTGTT CGGCCACACA GCCGAAAAAT 240

CCGGCCAGAT CATTATTACT ACAATTGAAA TATCATTGCA AGAAACAGAT CGGAGAAGAT 300

TTCATAGATA ATTTTGAACA GCTAAGTAAT AATATATATT TAAAGACAAT TTCTAGCCAT 360

TTAAAAAAGG TTTTTAGAAG GGATCCGGGC AAGTCCGGCG TCGACATCTA CCGGCTGATG 420

AAGTTCCAGC GCTCGAACCA GTCGACCTGC ATCAACACGT CCGCTGGTCA AGGTGGGCGA 480

CCGCATCGAG AAGGGCGACA TCATCGCTGA CGGTCCTTCG ACC 523

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM
(B) STRAIN: PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGTCGAAGGA CCGTCAGCGA TGATGTCGCC CTTCACGATG CGGTCGCCCA CCTTGACCAG 60

CGGACGTGTT GATGCAGGTC GACTGGTTCG AGCGCTGGAA CTTCATCAGC CGGTAGATGT 120

CGACGCCGGA CTTGCCCGGA TCCCTTCTAA AAACCTTTTT TAAATGGCTA GA@ATTGTCT 180

TTAAATATAT ATTATTACTT AGCTGTTCAA AATTATCTAT GAAATCTTCT CCGATCTGTT 240

TCTTGCAATG ATATTTCAAT TGTAGTAATA ATGATCTGGC CGGATTTTTC GGCTGTGTGG 300

CCGAACAACG GTCCGGCAGC GCTGGTTTCG CCTTCAAGTC GATGCAGCCG TGGCCGGTGC 360

CGACCATCAA CACGCGCTGC TGCTGTCGTC CGGCGCTACG TTGACGGTTT CGCACCACGC 420

ACTGCGTGAA GATCTTCACT TTATTGCTCA ATCTGCTCCA AGAATATTCA GAGCTATTTT 480

AACTTGGCTA TTAAAAGAAG GTGGTCAACG TTGGCTAGAG ATC 523

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM
(B) STRAIN: PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATCTTCACGC AGTGCGTGGT 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: CRYPTOSPORIDIUM PARVUM

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTCAGCCGG TAGATGTCGA 20

CLAIMS.

1. A method for the detection and/or identification of C. parvum organisms, or nucleic acid sequences specific thereto, comprising determining the presence of the nucleic acid sequence SEQ ID No 1 and/or 2, in polynucleic acid present in or derived from a sample under investigation, and relating the presence thereof to the presence of C. parvum or polynucleic acid thereof.

2. A method for the detection and/or identification of C. parvum organisms, or nucleic acid sequences specific thereto, comprising hybridization probing polynucleic acid present in or derived from a sample under investigation with an oligonucleotide that is capable of specific hybridization with sequences chraracteristic of SEQ ID No 1 and/or 2 and relating the occurence of hybridization with the presence of C. parvum or polynucleic acid thereof.

3. A oligonucleotide hybridization probe comprising 10 or more consecutive bases of the sequences SEQ ID No 1 or 2.

4. A method for the detection and/or identification of C.parvum organisms or nucleic acid sequences specific thereto, comprising use of specific sequence amplification to amplify any SEQ ID No 1 and/or 2 present in a sample, or polynucleic acid derived therefrom, and relating the production of product to the presence of C.parvum or said nucleic acid.

5. A method as claimed in claim 4 wherein the specific sequence amplification is carried out using the polymerase chain reaction.

6. A method as claimed in claim 5 wherein the forward and reverse PCR primers have 5' end sequences of SEQ ID No 3 and 4 respectively.

7. A polymerase chain reaction primer capable of acting as a primer

for amplifying SEQ ID No 1 and/or 2 and comprising an oligonucleotide of 5' end sequence of sequence SEQ ID No 3 or 4.

8. A polymerase chain reaction primer having an oligonucleotide sequence of SEQ ID No 3 or 4.

9. A method for the detection and/or identification of C.parvum or C.baileyi organisms, or nucleic acid sequences specific thereto, comprising determining the presence of the C. parvum nucleic acid sequence SEQ ID No 5 and/or 6, or the equivalent sequence present in C. baileyi, in polynucleic acid present in or derived from a sample under investigation, and relating the presence thereof to presence of one of these organisms or their polynucleic acid.

10. A method for the detection and/or identification of C. parvum or C. baileyi organisms, or nucleic acid sequences specific thereto, comprising hybridization probing polynucleic acid, present in or derived from a sample under investigation, with an oligonucleotide or polynucleotide probe that is capable of specific hybridization with a polynucleic acid sequence characteristic of polynucleic acid comprising a sequence SEQ ID No 5 and/or 6, other than SEQ ID No 1 and/or 2, and relating the occurrence of hybridization to the presence of said organisms or polynucleic acid.

11. A method as claimed in claim 10 wherein the probe is of SEQ ID No 5 and/or 6.

12. A method for the detection and/or identification of C. parvum or C. baileyi organisms, or nucleic acid sequences specific thereto, comprising determining the presence of the C.parvum nucleic acid SEQ ID No 7 and/or 8, or the equivalent sequence present in C. bailyei, in polynucleic acid present in or derived from a sample, and relating that to the presence of said organisms or nucleic acid.

13. A method as claimed in claim 12 wherein the polynucleic acid is probed with an oligonucleotiode or polynucleotide capable of specifically hybridizing with a sequence characteristic of SEQ ID No 7 and/or 8, or the equivalent sequences found in C. baileyi, and the occurence of hybridization is related to the presence of said organisms or nucleic acid.

14. An oligonucleotide or polynucleotide probe comprising any 10 or more consecutive bases of SEQ ID No 7 and/or 8, and not being of SEQ ID No 1 and/or 2.

15. A polynucleotide probe of SEQ ID No 7 and/or 8.

16. A method for the detection and/or or identification of C.parvum or C.baileyi organisms or nucleic acid sequences specific thereto comprising

(a) mixing a sample suspected of comprising said organisms and/or nucleic acid sequences with specific sequence amplification reaction reagents and primers for said amplification, said primers being targeted to specifically amplify a polynucleotide sequence comprising polynucleotides of SEQ ID No 5 and/or 6, or the equivalent sequence present in C. baileyi;

(b) subjecting the mixture to conditions under which the amplification of any sequence comprising SEQ ID No 5 and/or 6 present will occur;

(c) relating the production of any amplification product to the presence of C. parvum or C. baileyi organisms or nucleic acid sequences specific thereto.

17. A method as claimed in claim 16 wherein the specific sequence amplification reaction is the polymerase chain reaction and the primers used have 5' end sequences SEQ ID No 9 and 10.

18. A method as claimed in claim 17 wherein the primer sequences consist of sequences SEQ ID No 9 and 10.

19. A method as claimed in any one of claims 16, 17 or18 wherein the product polynucleotide is identified by application of a specific sequence amplification reaction to the product using primers targeted at SEQ ID No 1 and/or 2 and relating production of further product to the presence of C. parvum organisms or polynucleic acid and lack of further product to presence of C. baileyi organisms or polynucleic acid.

20. A method as claimed in claim 16, 17 or 18 wherein the product polynucleotide is identified by means of hybridization probing using a hybridization probe comprising sequences as claimed in claim 3, 14 or 15.

21. A method as claimed in claim 20 wherein the hybridization probe is specific for SEQ ID No 1 and/or 2, but not for the equivalent sequence of C. baileyi, and occurrence of specific hybridization is related to the presence of C. parvum or its specific polynucleic acids.

22. A polymerase chain reaction primer consisting of an oligonucleotide having a 5' end sequence of SEQ ID No 3, 4, 9 or 10.

23. A primer as claimed in claim 22 consisting of an oligonucleotide of SEQ ID No 3, 4, 9 or 10.

24. A method as claimed in any one of the preceding method claims incorporate a procedure for liberating nucleic acids from said organism before initiating amplification chain reaction conditions.

25. A method as claimed in claim 26 wherein the sample to be tested is either sonicated or subjected to freeze/thaw cycles in order to

rupture the organisms and liberate the nucleic acids.

26. A method as claimed in claim 24 or 25 wherein the organisms are first suspended in a medium.

27. A method as claimed in any on of claims 24 to 26 wherein the organisms are in the form of oocysts.

28. A probe as claimed in claim 3, 14 or 15 wherein the oligonucleotide is labelled by incorporation of a radioactive, chemical or biological label into its structure.

29. A diagnostic kit comprising one or more probes or primers as claimed in any one of claims 3, 7, 8, 14, 15, 22, 23 or 28.

30. A diagnostic kit as claimed in claim 29 further comprising metal tagged antibodies to cryptosporidium oocysts.

31. A diagnostic kit as claimed in claim 29 or 30 further comprising a reducing agent suitable for use in a method as described in Example 3 herein.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE, BOSTON, MASSACHUSETTS, USA, DECEMBER 1-5, 1991. AM J TROP MED HYG 45 (3 SUPPL.). 1991. 229. CODEN: AJTHAB ISSN: 0002-9637 13 - (C) FILE BIOSIS LAXER M A ET AL 'DETECTION OF ***CRYPTOSPORIDIUM*** -PARVUM DNA IN FIXED PARAFFIN-EMBEDDED TISSUE BY THE ***POLYMERASE*** ***CHAIN*** ***REACTION***.' see abstract --- -/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1993	26. 11. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	MOLINA GALAN, E

Form PCT/ISA/210 (second sheet) (July 1992)

Int ional Application No
PCT/GB 93/01559

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE vol. 45, no. 6 , 1991 pages 688 - 694 LAXER ET AL. 'DNA sequences for the specific detection of Cryptosporidium parvum by PCR' see the whole document	
A	BIOCHIM BIOPHYS ACTA 1131 (3). 1992. 317-320. CAI J ET AL '***PCR*** CLONING AND NUCLEOTIDE SEQUENCE DETERMINATION OF THE 18S RRNA GENES AND INTERNAL TRANSCRIBED SPACER 1 OF THE PROTOZOAN PARASITES ***CRYPTOSPORIDIUM*** -PARVUM AND ***CRYPTOSPORIDIUM*** -MURIS.'	

Return to PatentWeb top page

Order Reference Number: 773217

RETRIEVE PATENTS:
w9402635(A1)
1999/04/19 18:27:27 GMT: ORDER SUBMITTED
1999/04/19 18:27:29 GMT: Preparing patent number w9402635(A1), 28 pages
1999/04/19 18:28:17 GMT: ORDER READY

Delivery mode: Download PDF
Patent number w9402635(A1), 28 pages: **$ 4.95**

Order Total: $ 4.95

Connecticut residents must add 6% sales tax.

Please Note:

Download orders can take some time to prepare, normally from fifteen seconds to fifteen minutes, depending on the size of the order and the PatentWeb™ system load. Return to this screen and click "Current Status" to see how the preparation of your order is progressing. The status screen that informs you that your order is ready will also present you with hypertext links to the individual patents in your order, allowing you to download them as .pdf files for viewing directly in the PDF viewer associated with your browser.

The preparation of your order will proceed even if your session expires or is disrupted. There is no need to re-enter your order. Once ready, your order will be retrievable for approximately 36 hours via the "Retrieve Orders" button on the PatentWeb™ main screen.

Occasionally, the retrieval of an ordered patent may be unsuccessful due to a timeout somewhere in the communications chain between MicroPatent® and the user's machine. Repeated attempts at retrieval are usually ultimately rewarded. If the problem persists, you should discuss it with us and with your Internet service provider.

Return to PatentWeb top page